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TITLE X-RAY HOLOGRAPHY OF BIOLOGICAL SPECIMENS

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X-RAY HOLOGRAPHY OF BIOLOGICAL SPECIMENS

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ABSTRACT

I review the reasons for x-ray imaging of biological specimens and the techniques presently being used for x-ray microscopy. I point out the advantages of x-ray holography and the difficulties of obtaining the requisite coherence with conventional sources. I discuss the problems of radiation damage and the remarkable fact that short pulse x-ray sources circumvent these problems and obtain high-resolution images of specimens in the living state. Finally, I review some of the efforts underway to develop high-intensity coherent x-ray sources for the laboratory.

WHY X-RAYS?

The most obvious reason for extending the art of microscopy into the x-ray regime is to improve resolution. By any criterion, the resolution of a microscope cannot exceed the wavelength of the probe radiation. In the 1930s, scientists realized that electrons could be used in lieu of photons. Electron deBroglie wavelengths are short even at modest energies, and electron microscopy radically improved resolution. Ultimately it allowed the examination of specimens on the atomic level. However, there are certain problems with using electrons as a probe.[1] First, they cannot deeply penetrate the specimen without loss of resolution owing to multiple Coulomb scattering. Second, specimen damage is severe owing to the high quantum energy of the electrons. Short wavelength photons do not suffer from the multiple scattering problem and, as will be shown later, short pulses can circumvent the damage problem. X rays can also obtain high contrast without staining, which is usually necessary with electron microscopy.[1,2] This is illustrated by Figure 1, which shows x-ray absorption length versus x-ray quantum energy. The maximum contrast between the proteins, which comprise the main structural

elements of a living cell, and water, which is the primary constituent of the cytoplasm, occurs between the K edges of carbon and oxygen. The greatest contrast is near the K edge of nitrogen.

X-RAY IMAGING TECHNIQUES

Figure 2 illustrates three x-ray imaging techniques that are in general use today.

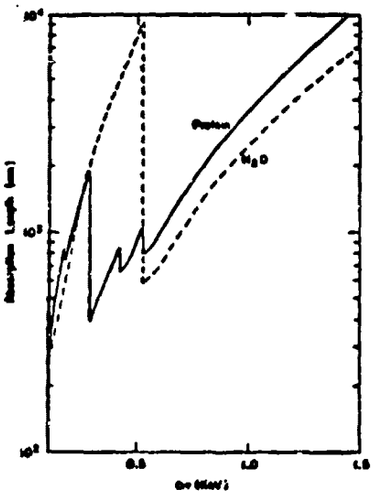

Figure 1

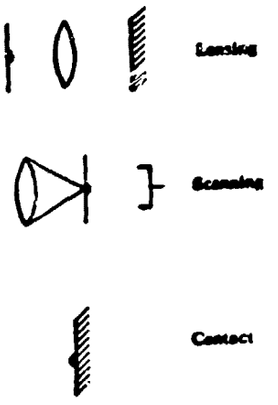

Figure 2

Lensing microscopy is being developed by a number of researchers, most notably by G. Schmahl[3] et al. in Germany. This kind of x-ray microscope is analogous to an optical microscope. The specimen is back-lighted with an x-ray source, e.g. a synchrotron light source, and an x-ray lens is used to focus the image on a recording medium, which can be ordinary x-ray film. Because indices of refraction are very small in the x-ray regime, it is not possible to use a refractive lens. We must substitute a diffractive lens, called a Fresnel zone plate, which consists of a set of concentric circles of opaque material that form a cylindrical diffraction grating that focuses monochromatic radiation to a point. A problem with lensing microscopy is that its resolution is limited to the finest grating spacing of the zone plate. Thus we must fabricate the zone plate to a precision comparable to the microscope's ultimate resolution. A second problem with lensing microscopy is that it inefficiently collects the

x rays scattered from the specimen. Consequently, the specimen must suffer high radiation doses to obtain an image of reasonable quality. At present, zone plate fabrication technology is limited to grating spacings of about 500 Å, but the technology is rapidly improving.

In scanning microscopy, the lens is used to focus the x-ray beam to a point on or within the specimen. The specimen is translated in a scanning pattern and a high-quantum-efficiency detector is used to register the transmitted portion of the x-ray beam. The detector signal is digitized and fed to a computer along with the scanning position. Scanning inflicts less damage than lensing and wet cell experiments have already given low-resolution pictures of algae in the living state.[4] Scanning also allows a broad range of uses of the probing radiation, for example, the specimen can be imaged in fluorescent radiation as well as in absorption or scattering. At present, scanning x-ray microscopy is being used by J. Kirz et al. at the NSLS in Brookhaven. They obtain specimen images with a resolution of about 1/5 μm, and ultimately they expect to improve this resolution to a limit of about 200 Å.

Contact microscopy is so named because it places the specimen in direct contact with the recording medium. X rays produce a shadow of the specimen. There is no magnification so a very high resolution recording medium such as photoresist is required. Photoresists are polymers that can be made locally less resistant to etching by exposure to radiation. After the specimen in contact with the photoresist is exposed to the x-ray source, the resist is developed in a strong base. The result is a contour plot of the local opacity of the specimen on the surface of the photoresist. This can then be examined by an electron microscope to obtain information about the structure of the specimen in the x-ray regime. The technique has been brought to a high level of sophistication by R. Feder[5] et al. who have obtained resolutions between 50 and 100 Å. The contact technique can resolve only those features of the specimen in intimate contact with the photoresist. Furthermore, diffraction around fine features of the specimen changes the direction of propagation of

x rays into the photoresist, so resolution is lost if the resist is deeply etched.[6]

WHY HOLOGRAPHY?

Holography is lensless: it requires no high-precision zone plate. Also, the channels of exposure in the recording medium are nearly normal to the recording surface, so it does not suffer from the diffraction problems of contact microscopy. Furthermore, holography produces fully three-dimensional images. These are not stereoscopic pictures but contain all the information of the specimen's structure within resolution limits. The importance of three-dimensional structural information has become increasingly clear, perhaps best exemplified by the heroic efforts to map the synaptic junctions in nerve tissue.

WHAT HOLOGRAPHY COSTS

Given the advantages of holography over other x-ray microimaging techniques, why is it not in general use? Because it requires coherence, and most x-ray sources are not coherent. We can obtain coherence from an incoherent source such as a synchrotron, by sacrificing intensity. Spacial coherence can be obtained with pinhole and temporal coherence can be obtained with a monochrometer. Both of these reduce intensity and increase exposure time. Nevertheless, there have been a number of successful attempts to obtain holograms of biological specimens with varying degrees of resolution.

TYPES OF HOLOGRAPHY

Figure 3 illustrates the technique known as Gabor, or on-axis Fresnel-transform holography. The same beam of coherent x rays provides specimen illumination and reference waves. The hologram is generated by the interference of the reference waves with the scattered waves at the recording surface. Because both intensity and phase information are recorded, it is possible, by the symmetry properties of Maxwell's equations, to reproduce a fully three-dimensional image.

TYPES OF HOLOGRAPHY

Gabor holography has two problems: (1) on reproduction, both a real and virtual images are generated and these can obscure each other, and (2) resolution is limited to twice the grain size of the recording medium. The double-image problem can be mitigated by placing the recording surface far from the specimen, but this moves the image back in the field of view and makes it more difficult to examine. If the information on the recording surface is directly digitized, it is possible to reconstruct the image with a computer and to extract the real and virtual images separately. In principle, this can be done with a minimum increase in entropy. Photoresists could provide the necessary high-resolution recording medium, and the image could be easily digitized using a tunneling electron microscope.

Figure 4 illustrates Leith-Upatnieks holography, or off-axis Fresnel transform holography. Its only advantage over Gabor holography is that it separates the real and virtual images upon reconstruction.

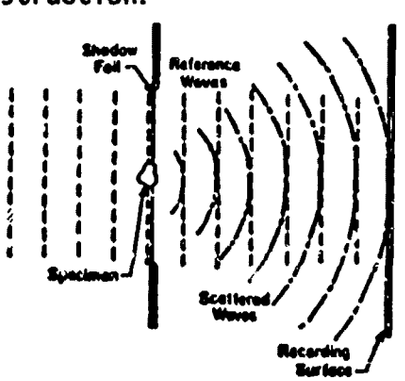

Figure 3

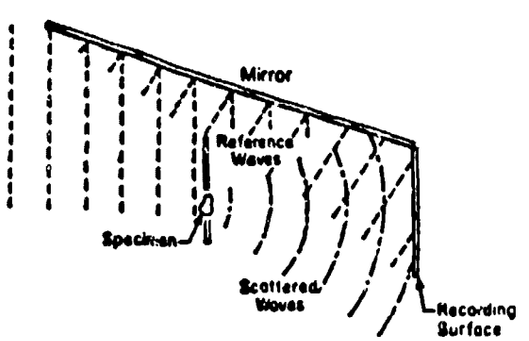

Figure 4

Figure 5 illustrates a holographic technique that requires less spacial and temporal coherence and is not limited by recording surface resolution. It is called Fourier transform holography and was invented by G. Stroke. It uses curved rather than planar reference waves so that spacial dimensions in the specimen translated to spacial frequencies on the recording surface. However, there is difficulty in obtaining the curved reference waves at sufficient intensity. If this is done with a zone plate, the resolution is limited

to the finest spacing of the grating, just as for the lensing microscopy techniques.

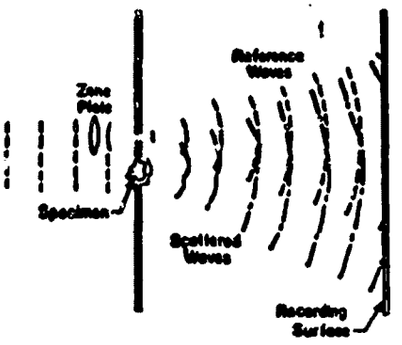

Figure 5

SPECIMEN DAMAGE

I have noted that holography derives from the symmetry properties of Maxwell's equations. That is 19th century physics. We now know that radiation is not described by continuously measurable values of electric and magnetic field but in fact occurs in quanta that we call photons. Thus any form of microscopy is an inherently statistical process. Statistical fluctuations go unnoticed in a light microscope because the quantum energy of the individual photons is small. In the x-ray regime, however, we must consider counting statistics to determine resolution. The radiation dosage depends on the resolution we are seeking. Table 1 gives a list of specimens and the radiation dosage necessary to obtain 100 Å resolution at a signal-to-noise ratio of five using probe radiation at the K edge of nitrogen. For technical reasons, holography requires slightly more exposure than microscopy,[6] but, as seen from the table, both techniques require tremendous dosages. While there are some bacteria that can survive enormously high dosages,[7] in general, the unperturbed living state cannot be preserved.

There is a technique that can circumvent the radiation dosage problem and obtain an image of the specimen in the living state.[6,8] This is to make the exposure very brief — a snapshot. The specimen will be severely damaged, in most cases it will explode. But the image can be extracted from the plasma ghost of the exploding specimen before its features have moved far enough to cause blurring. Table 2 shows some conservative estimates of the intensities and

SPECIMEN DAMAGE

TABLE 1

Intensity and Exposure Times

(I) SPECIMEN				
Resolution (nm)	10	10	20	20
Globule Radium (nm)	2.5	50	2.5	50
(II) CONTACT/LENS				
Intensity ($w \cdot cm^{-2}$)	3.1×10^{10}	1.0×10^{10}	2.5×10^{8}	2.2×10^{8}
Exposure Time (ns)	3.1×10^{-2}	3.0×10^{-2}	4.9×10^{-1}	1.7×10^{-1}
(III) MICROHOLOGRAPHY				
Intensity ($w \cdot cm^{-2}$)	5.7×10^{15}	1.8×10^{13}	3.7×10^{15}	1.4×10^{11}
Exposure Time (ns)	5.5×10^{-4}	2.5×10^{-3}	2.0×10^{-3}	2.0×10^{-2}

TABLE 2

Dosage (rad) to Obtain 100 Å Resolution at S/N = 5

		($h\nu$ = 400 eV, ε = 1)	
Specimen	Mass (g)	Microscopy	Holography
Escherichia coli phage ϕ×174	10^{-17}	2×10^{12}	2×10^{12}
Herpes viron	10^{-16}	2×10^{12}	2×10^{12}
Vaccinia viron	10^{-15}	6×10^{13}	6×10^{13}
Mycoplasma pneumoniae	10^{-14}	3×10^{9}	3×10^{9}
Escherichia coli (immature)	10^{-13}	3×10^{9}	3×10^{9}
Escherichia coli (mature)	10^{-12}	3×10^{9}	3×10^{9}
Anthrax bacterium	10^{-11}	3×10^{9}	4×10^{9}
Red blood cell (human)	10^{-10}	4×10^{9}	5×10^{9}
White blood cell (human)	10^{-9}	3×10^{9}	4×10^{9}
Amoeba (dysentery)	10^{-8}	2×10^{9}	2×10^{9}
Smooth muscle cell	10^{-7}	3×10^{10}	4×10^{10}
Paramecium (protozoan)	10^{-6}	1×10^{18}	2×10^{18}

exposure times required to obtain various resolutions of exploding globules of protein in water. Note that while the total energies required are modest, the pulses are extremely short and the intensities extremely high. Fortunately, short pulses and high intensities are obtained easily from laser-like sources. It is impossible to obtain such pulses from electron sources because of space charge.

X-RAY SOURCES

Two properties of the x-ray source are necessary for microholography of biological specimens in the living state: (1) high coherence, and (2) short pulses. Both of these properties are easily obtained from lasers. Because excited state lifetimes are short and cross sections are small, an x-ray laser will require a high-energy-density source to drive it. One possible high-energy-density device is a nuclear explosive. It is an unclassified fact that the DOE Laboratories are working on nuclear explosive-driven x-ray lasers, but that is all that is unclassified. Fanciful[9,10] reporters have projected images of biologists carrying their specimens to the Nevada Test Site for elaborate experiments involving nuclear explosive-driven x-ray lasers. This will simply not happen. Everything related to nuclear-explosive-driven x-ray lasers, other than that they are being pursued, is classified and is likely to remain so.

Another high-energy density source is the focus of a high-energy short-pulse laser. The Lawrence Livermore Laboratory has reported some success with this approach.[11] It appears that researchers at Livermore have demonstrated lasing in the 3P to 3S transition of neon-like selenium. By neon-like we mean selenium atoms that have been stripped of all but 10 electrons leaving their electronic configuration very much like that of neon. While the quantum-mechanical description of the electron orbitals is very similar to neon, the quantum energy of transitions is shifted upward owing to the higher effective charge of the nucleus. The lasing 3P to 3S transition of neon-like selenium has a quantum energy of about 70 eV. While this is still somewhat far from the K edge of carbon (250 eV), it is still

a major step toward the realization of laboratory x-ray holography. Livermore researchers also working on higher quantum-energy lasers, in particular those involving 3 to 2 transitions in helium-like neon and helium-like fluorine. If successful, these lasers should produce quantum energies of about 150 eV. Los Alamos scientists have also proposed x-ray lasers to be pumped by laboratory lasers, in particular to be pumped by the hot-electron spectrum produced by CO_2 lasers.[12]

Another approach is to produce a coherent source of high-quantum energy radiation from the harmonics of high-intensity lasers. At this writing, the shortest wavelength obtained by such a technique was from the 7th harmonic of a KrF laser producing radiation at about 350 Å.[13] Scientists at the University of Illinois have produced a source from the 5th harmonic of ArF which has a wavelength of about 390 Å.[14] In general, such harmonic generators, or virtual state sources, suffer from decreasing efficiencies as the order of the harmonic is increased. For example, the short-pulsed lasers in nonlinear media produce a 3rd harmonic at efficiency of about 10^{-5}, the 5th harmonic at an efficiency of about 10^{-7} and the 7th harmonic at an efficiency of about 10^{-10}.

An alternative is to use multiphoton pumping of real states. C. Rhodes et al. have demonstrated an inner-shell laser by 4-photon pumping of Kr.[15] This multiphoton pump laser produces radiation in 5 lines, the shortest wavelength of which is 916 Å. The laser operates at a few μJ with efficiency of about 10^{-4}. This technique of playing the quantum-mechanics game cleverly and utilizing high-order multiphoton techniques seems very promising. Recently, Rhodes et al. demonstrated 99 photon ionization of uranium. This correspondends to a quantum energy of 630 eV. If a technique can be found for channeling this high-order multiphoton pumping into a lasing transition, certainly the wavelengths necessary for x-ray holography of biological specimens will be obtained.

If the present trends in technology continue, I believe the first demonstration of laboratory short-pulse x-ray holography will occur before the end of the decade, and x-ray holographs will become as common as electron microscopes by the turn of the millennium.

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